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Protective Role of Antimannan and Anti-Aspartyl Proteinase Antibodies in an Experimental Model of *Candida albicans* Vaginitis in Rats

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The role of antibodies (Abs) in the resistance to vaginal infection by *Candida albicans* was investigated by using a rat vaginitis model. Animals receiving antimannoprotein (anti-MP) and anti-aspartyl proteinase (Sap) Ab-containing vaginal fluids from rats clearing a primary *C. albicans* infection showed a highly significant level of protection against vaginitis compared to animals given Ab-free vaginal fluid from noninfected rats. Preabsorption of the Ab-containing fluids with either one or both proteins MP and Sap sequentially reduced or abolished, respectively, the level of protection. A degree of protection against vaginitis was also conferred by postinfectious administration of anti-Sap and anti-MP monoclonal antibodies (provided the latter were directed against mannan rather than protein epitopes of MP) and by intravaginal immunization with a highly purified, polysaccharide-free Sap preparation. Postinfectious administration of pepstatin A, a potent Sap inhibitor, greatly accelerated the clearance of *C. albicans* from rat vagina. No anti-MP or anti-Sap Abs were elicited during a *C. albicans* vaginal infection of congenitally athymic nude rats. Although they were as able as their euthymic counterparts to clear the primary infection, these animals did not show increased resistance to a rechallenge, demonstrating that induction of anticandidal protection in normal rats was a thymus-dependent Ab response. Overall, our data strengthen the concept that Abs against some defined *Candida* antigens are relevant in the mechanism of acquired anticandidal protection in vaginitis. The T-cell dependence of this protection may also provide a link between cell-mediated and humoral immunity in vaginal infection.

Vulvovaginal candidiasis is a widespread, common disease affecting about one third of all women at least once in their lifetimes; about 5% of these women experience recurrent attacks of the disease (21, 41). The pathogenesis of this frequent clinical problem remains unknown. Since the incidence of mucosal candidiasis is higher in patients with impaired cell-mediated immunity (CMI), including AIDS patients (28), it is rather commonly held that susceptibility to vaginal infection by *Candida* is controlled by CMI. In a series of studies, Fidel and collaborators have suggested that local rather than systemic CMI is an important host defense mechanism of the vaginal mucosa (17–20). However, the nature of the immunoprotective factors remains undefined.

In order to understand the fungal and host components involved in the pathogenesis of candidal vaginitis and immune responses to *Candida albicans*, the primary agent of this disease, we have long been studying a rat model of vaginal infection (11–14). In this model, we have recently addressed the potential role of antibodies (Abs) in anti-*Candida* immunity and protection at the vaginal level. Assuming that *C. albicans* infection is essentially extracellular, we reasoned that if adherence, germ tube formation, and production of putative virulence enzymes, such as aspartyl proteinase(s) (Sap) play a role in infection (2, 4, 10, 26, 29, 40), it is conceivable that specific Abs against these factors could be protective. This approach has recently been substantiated by the observation that, after clearing the primary *C. albicans* vaginal infection, animals acquired Ab-mediated resistance to vaginal reinfection with the fungus (7). However, the specificity of the anticandidal Abs in the vaginal mucosa was not precisely determined.

Thus, we (i) further investigated the role of specific Abs in protection, (ii) examined the use of monoclonal reagents directed against various molecular constituents of the fungal cell, and (iii) determined whether the protective Ab response at the vaginal level was directed against thymus-dependent antigens.

MATERIALS AND METHODS

Microorganism and growth conditions. The yeast used throughout this study was *C. albicans* SA-40, isolated from the vaginal secretion of a patient suffering from acute vaginitis (6). For the experimental infection, a subculture of the strain from Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) was grown in yeast extract-peptone-dextrose medium (yeast extract, 1% [wt/vol]; neopeptone, 2% [wt/vol]; dextrose, 2% [wt/vol]) for 24 h at 28°C in a shaker (200 rpm), harvested by centrifugation (3,500 × *g*), washed, and suspended to the required number in phosphate-buffered saline (PBS; Oxoid, Unipath Ltd., Basingstoke, England).

Animals. Oophorectomized euthymic and athymic "nude" Rowett rats (body weight, 80 to 100 g) (Charles River Breeding Laboratories, Calco, Italy) were used throughout this study. Animal maintenance, estrogen treatment, and modalities of both the primary infection and the rechallenge were as described elsewhere (7, 12).

Experimental rat vaginitis. All rats were maintained under pseudoestrus by administration of estradiol benzoate (Benzatrone; Samil, Rome, Italy) every 2 days. Six days after the first estradiol dose, each animal was inoculated intravaginally with 10^7 yeast cells of *C. albicans* in 0.1 ml of PBS administered with a syringe equipped with a multipurpose calibrated tip (Combitip; PBI, Milan, Italy). The cells in the vaginal fluid were counted by culturing 1-μl samples of vaginal fluid (taken from each animal by a calibrated plastic loop [Disponoic; PBI]) on Sabouraud agar containing chloramphenicol, at 28°C for 72 h. One

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vaginal sample per rat was evaluated, and a rat was considered infected when at least 1 CFU was present in one loop of vaginal fluid, i.e., a count of $\geq 10^3$ CFU/ml (11).

For Ab analysis, samples of vaginal fluids were taken at regular intervals from each animal by gently washing the vaginal cavity with 0.5 ml of PBS, as described elsewhere (7). The collected fluid was centrifuged at $3,500 \times g$ for 15 min in a refrigerated Biofuge, and the supernatant was assayed as described below.

ELISA to detect Abs against *C. albicans* constituents in vaginal fluids. The presence of anti-*Candida* Abs in the vaginal washes was assayed by a previously described enzyme-linked immunosorbent assay (ELISA) (7). Briefly, 200 μl of a mannoprotein (MP) extract (4, 43) solution (5 μg/ml in 0.2 M sodium carbonate) was used as coating antigen by dispensing it into the wells of a polystyrene microtitration plate which was kept at 4°C overnight. After three washes with Tween 20-PBS buffer, twofold dilutions of vaginal fluids were distributed in triplicate wells, and the plates were incubated for 1 h at room temperature. Each well was washed again with Tween 20-PBS buffer, and predetermined optimal dilutions of alkaline phosphatase conjugate with sheep anti-rat immunoglobulin G (IgG), IgM, or IgA (Serotec Ltd., Kidlington, Oxford, United Kingdom) were added. Controls were plates without any coating antigen. Bound alkaline phosphatase was detected by the addition of a solution of *para*-nitrophenyl phosphate in diethanolamine buffer, and the plates were read at A_{405} with an automated microreader (Labsystem Multiscan, Helsinki, Finland) blanked against air.

For anti-Sap Ab detection, the same ELISA was used, except that a highly purified, non-mannan-containing Sap preparation (Sap2; kindly provided by P. A. Sullivan, Dunedin, New Zealand) was used. The preparation was checked for the degree of enzyme purification as described by Ross et al. (38), the presence of its characteristic single electrophoretic band was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and its identity was confirmed by Western blotting with a specific anti-Sap2 serum (11, 38). Vaginal fluid was considered positive for a determined Ab when the optical density was greater than 2 standard deviations from the mean value for the well coated with the same antigen and for the assay of the irrelevant Ab-negative vaginal fluid from an uninfected rat (7).

Active immunization. Five rats under the usual estrogen treatment (see above) were immunized intravaginally with 100 ng of Sap which was emulsified in 0.1 ml of complete Freund adjuvant (Difco Laboratories) (days 0 and 7) or suspended in PBS (days 14 and 21). Two other groups of five rats each received the same volume of adjuvant or PBS only. On day 30, all animals, under the usual estrogen treatment, were challenged intravaginally with 10^7 cells of *C. albicans*, and vaginal CFU were enumerated as described above. The experiment was repeated, and vaginal fluids were harvested for assessment of anti-Sap Abs.

Absorption of immune vaginal fluid with *C. albicans* constituents. Vaginal fluids containing anti-MP and anti-Sap Abs were separately absorbed onto nitrocellulose sheets precoated with 5 μg of MP or Sap or 10 μg of capsular polysaccharide of *Bacteroides fragilis* as a control. This was referred to as the irrelevant antigen and was kindly offered by A. Pantosti, Laboratory of Bacteriology and Medical Mycology, ISS, Rome, Italy. Some immune vaginal fluids containing anti-MP and anti-Sap Abs were tested with both antigens sequentially. The presence of anti-MP and anti-Sap Abs was checked by ELISA before and after absorption. Vaginal fluids from noninfected animals were also used as a control.

In vivo treatment with pepstatin. Pepstatin A (P 4265; Sigma Chemical Co., St. Louis, Mo.) was dissolved in 95% ethanol, diluted 1:50 in sterile H_2O, and administered intravaginally at a final concentration of 100 μg in 0.1 ml to a group of five estrogen-treated rats which had been infected (30 min before pepstatin administration) with the usual challenging dose of 10^7 *C. albicans* cells. Control rats received pepstatin diluent or PBS only. Treatment with the drug or the diluent was continued daily for 7 days.

Treatment with monoclonal antibodies. The monoclonal Abs (MAbs) used throughout this study were MAb AF1 (IgM), which recognizes a polysaccharide epitope of *C. albicans* MP (5); MAbs 4C8 and 8E11 (both IgG1), which recognize two distinct polypeptide components of MP (23); and GF1 (IgG1), which is directed against the Sap2 of *C. albicans* (unpublished data). Before use, the specificities were checked by Western blotting, and the MAbs were intravaginally administered at identical protein concentrations (100 μg/ml). All MAbs were purified on protein A affinity columns as described elsewhere (32).

Passive transfer of Abs. Vaginal washes were collected by the method described above from rats which had cleared a primary vaginal infection with *C. albicans* (7). Uninfected rats were also used as donors of nonimmune vaginal fluid. Either unadsorbed or antigen-adsorbed fluids (see above) were brought to identical protein concentrations, and 0.5 ml of each fluid was administered intravaginally into naive, nonimmunized, oophorectomized, and estradiol-treated rats. After 30 min, each recipient rat was inoculated with *C. albicans* at the usual challenging dose of 10^7 cells, and the number of *C. albicans* vaginal CFU was determined as described above.

Effector cell preparations. The spleens of euthymic and athymic rats were aseptically removed and gently minced in lysis buffer (0.16 M Tris-buffered NH_4Cl, pH 7.2), and the cellular debris was removed. The cell suspensions were counted and diluted for proliferation assays to an appropriate concentration (2×10^5/ml) in RPMI 1640 medium (Flow Laboratories, Irvine, United Kingdom) containing 10% heat-inactivated fetal calf serum, 2 mM L-glutamine (Gibco Laboratories, Grand Island, N.Y.), 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 20 mM HEPES (complete medium; Gibco).

Heparinized venous peripheral blood was taken aseptically by cardiac puncture from CO_2-anesthetized rats. Peripheral blood mononuclear cells were isolated by centrifugation on Ficoll-Hypaque (Lymphoprep; Nicamed, Oslo, Norway) gradients, washed twice, counted, and finally resuspended at the appropriate concentration in complete medium for the cytofluorimetric assay.

Cell proliferation. Peripheral blood mononuclear cells and spleen cells were dispensed into tissue culture-treated, plastic, 96-well, round-bottomed microtiter plates (Costar, Cambridge, Mass.) at a final concentration of 10^5 cells/0.1 ml and cultured in a total volume of 200 μl of culture medium with different concentrations of concanavalin A (5.0, 2.5, and 1.25 μg/ml), phytohemagglutinin (2.0, 1.0, and 0.5 μg/ml), and human recombinant interleukin-2 (hrIL-2; 500, 250, and 50 IU/ml) for 72 h or MP (100, 50, and 10 μg/ml) for 7 days, at 37°C in a 5% CO_2 humidified atmosphere.

Cell proliferation was evaluated by thymidine (TdR) incorporation (0.5 μCi of [^{3}H]TdR per well; 6.7 Ci/mmol; New England Nuclear, Boston, Mass.) during the last 6 h of a 72-h culture. MP-induced cell proliferation was measured as described above, but [^{3}H]TdR was added during the last 18 h of a 7-day culture. Incorporation of [^{3}H]TdR was measured by standard liquid scintillation counting techniques after the cells were harvested with the Skatron (Oslo, Norway) harvester. All cultures were run in quadruplicate.

Immunofluorescence and flow cytometric analysis. T-cell phenotype was evaluated by immunofluorescence and fluorescence-activated cell sorter analysis with the following murine MAbs: fluorescein isothiocyanate (FITC)-conjugated anti-rat CD4 and anti-T-cell receptor αβ and phycoerythrin-conjugated anti-rat CD5 and anti-CD8 (PharMingen, San Diego, Calif.). Natural killer (NK) phenotype was evaluated with a mouse FITC-conjugated anti-rat MAb (Serotec Ltd.) that recognizes NK-RP1, a triggering structure selectively expressed on rat NK cells (9). As CD8 antigen is expressed on both T and NK cells, $CD8^+$ T cells were evaluated by subtracting the number of NK-$RP1^+$ cells from the total number of $CD8^+$ cells. Phycoerythrin mouse IgG1 and FITC mouse IgG2a (PharMingen) served as negative controls.

Peripheral blood lymphocytes (PBLs) and spleen cells from Rowett nude rats were suspended in complete medium and incubated with the appropriate Ab or negative control for 30 min at 4°C. After three washes with cold PBS, the cells were analyzed for relative fluorescence intensity. The percentage of positive-stained cells determined for 10,000 events was analyzed on a FACScan cytofluorimeter (Becton Dickinson, Mountain View, Calif.). Fluorescence intensity was expressed in arbitrary units on a logarithmic scale.

Statistics. All differences in the mean numbers of CFU of *C. albicans* in vaginal fluids were assessed by Student's *t* test, while the differences in the ratios of the number of infected rats/total number of rats was evaluated by the Mann-Whitney U test. In both cases, the significance was set at a P value of <0.05 (two-tailed test).

RESULTS

Outcome of primary and secondary vaginal infections in congenitally athymic nude rats. In order to investigate whether the protection (and the Ab response) conferred upon rats by a primary *C. albicans* infection was thymus dependent, experimental vaginitis was reproduced in congenitally athymic nude rats. Before this investigation, we verified the phenotype and function of spleen and PBLs of the nude rats used throughout our experiments. Immunocytometric analysis of PBLs demonstrated that slightly less than 50% of the cells had the marker NK-RP1 and only 6% of the cells had the rat T-cell antigen CD5. Among the latter, <2% were $CD4^+$ and approximately 5% were αβ T-cell receptors. Spleen cells were only 2% $CD5^+$. Neither the PBLs nor the splenocytes proliferated in vitro to any appreciable extent in response to mitogens such as concanavalin A, phytohemagglutinin, or IL-2 (data not shown).

Table 1 shows the outcome of primary and secondary challenges of athymic rats with *C. albicans*. The animals showed a primary infection not dissimilar in extent and duration to that observed in euthymic rats (see below and reference 7). In fact, following an intravaginal inoculation with 10^7 yeast cells, $>1 \times 10^5$ to 1×10^4 *Candida* CFU/ml of vaginal fluid was found in the first week postinfection, and then the vaginal *Candida* burden slowly decreased to close to 10^3 cells by four weeks. Smears of vaginal fluids from rats taken at intervals during infection, starting at day 1, showed intense hyphal growth (not shown).

When, after clearance of the primary infection (40 days), the

TABLE 1. Outcome of primary and secondary vaginal infections in athymic rats[a]

Infection	Mean *C. albicans* vaginal CFU ± SE (no. of rats infected/total) on day[a]:					
	0	1	2	7	14	21
Primary	>100 (10/10)	94.5 ± 4.2 (10/10)	85 ± 3.5 (10/10)	47.2 ± 6.6 (8/10)	19.7 ± 4 (7/10)	16.2 ± 4.6 (4/10)
Secondary	>100 (10/10)	94.1 ± 6.7 (10/10)	74.1 ± 7.3 (10/10)	45 ± 8.7 (8/10)	16 ± 4 (6/10)	4 ± 2 (4/10)

[a] There were no statistically significant differences between primary and secondary infections at any time point for either CFU (data expressed as 10^3 CFU/ml) or the ratio of the number of infected rats to the total number of rats tested. Rats were considered infected when vaginal CFU counts were more than 10^3/ml (7).

same animals were rechallenged with 10^7 fungal cells, the infection was reestablished, with vaginal CFU counts equalling those during the primary infection and kinetics similar to those of *Candida* clearing (Table 1). This experiment, which was repeated twice with similar results, clearly demonstrated that the athymic rats which cleared a primary infection were not protected against a secondary fungal challenge.

Vaginal fluids from rats taken at intervals during both the primary and the secondary infections were evaluated for the presence of anti-MP or anti-Sap Abs. No such Abs were consistently found during either challenge. Only occasionally (two rats on day 7 and two on day 28 during the primary infection and another two rats on day 7 of the secondary infection) were vaginal fluids positive for the MP antigen only, but the response was low, and the Abs belonged exclusively to the IgM isotype.

As a control, an experimental infection in euthymic rats under the conditions selected for the present study was repeated. These animals, in contrast to the athymic ones, were essentially resistant to a second challenge with *C. albicans* ($<10^3$ candidal CFU within the first 5 days after challenge) (7). Consistent levels of both anti-MP and anti-Sap Abs (predominantly of the IgG class) were found in the euthymic rats' vaginal fluids, mostly during the secondary infection, confirming previous results (7).

Passive transfer of immunoprotection by vaginal fluids containing anti-MP and anti-Sap Abs. To assess the extent to which the vaginal anti-MP or anti-Sap Abs of euthymic rats were responsible for protection against a secondary challenge by *C. albicans* (7), we performed passive protection experiments whereby the immune vaginal fluids were adsorbed with MP or Sap or both and were then administered into the vaginal cavities of rats (single administration) 30 min before *C. albicans* challenge. The controls were rats receiving (i) *C. albicans* cells only, (ii) nonabsorbed immune vaginal fluid (thus containing both anti-MP and anti-Sap Abs and possibly other Abs), (iii) nonimmune vaginal fluid (without anti-MP and anti-Sap Abs), and (iv) immune vaginal fluid previously absorbed with an irrelevant antigen and still containing both anti-MP and anti-Sap Abs. Because the effect of passive transfer of a single Ab administration was manifested mostly in the initial stages of the infection (7, 36), Table 2 shows only the data collected during the first week after *C. albicans* challenge.

Control rats receiving only *C. albicans* cells had the usual (7, 11–13) fungal burden vaginally for the whole duration of the observation, similar to the animals which were pretreated with vaginal fluids from nonimmunized rats. In contrast, the animals receiving immune vaginal fluid containing both anti-MP and anti-Sap Abs showed a reduction of at least 50% in the number of CFU from the first day after infection, and the reduction persisted for one week, with P values always <0.05. A similar decrease in vaginal *C. albicans* burden was detected in animals given immune vaginal fluid preabsorbed on irrelevant antigen (Table 2).

The animals receiving vaginal fluid containing only anti-MP or anti-Sap Abs appeared to be less protected compared to those subjected to passive transfer of both Abs (especially considering the first day after challenge [Table 2]). However, they still eliminated the *Candida* cells more rapidly than the controls not given immune fluid, and their protective activity tended to equal that of animals given anti-Sap plus anti-MP Ab-containing vaginal fluid later during infection (day 7). Finally, the rats treated with vaginal fluid from which both anti-MP and anti-Sap Abs had been eliminated by sequential absorption on the respective antigens showed kinetics of vaginal infection similar to those of control rats that were not pretreated or were given Ab-free vaginal fluids.

Overall, the data showed that passive transfer of Abs confers protection against *Candida* challenge and suggest that both anti-MP and anti-Sap Abs contribute to the protection. The fact that no residual protection was conferred by vaginal fluids that were absorbed to remove anti-MP and/or anti-Sap antibodies also demonstrates that other unspecified Abs which

TABLE 2. Outcome of vaginal infection in rats subjected to passive transfer of various vaginal fluids before challenge with *C. albicans*

Type of pretreatment	Type of Ab present	Mean *C. albicans* vaginal CFU ± SE (10^3/ml) on day[a]:			
		0	1	2	7
None	None	>100	>100	98 ± 2	47 ± 3
Nonimmune vaginal fluid	None	>100	>100	>100	62 ± 4
Immune vaginal fluid[b]	Anti-MP, anti-Sap	>100	50 ± 5*	43.4 ± 5*	22 ± 3*
Immune vaginal fluid after absorption on irrelevant antigen	Anti-MP, anti-Sap	>100	44 ± 7*	60.2 ± 8.4*	22 ± 4*
Immune vaginal fluid after absorption on MP	Anti-Sap	>100	77 ± 8*	66 ± 3.5*	30 ± 4*
Immune vaginal fluid after absorption on Sap	Anti-MP	>100	60 ± 6.5*	58 ± 9*	30 ± 5*
Immune vaginal fluid after absorption on Sap and MP	No anti-MP, no anti-Sap, possibly other unknown Abs	>100	>100	>100	65 ± 5

[a] There was a statistically significant difference ($P < 0.05$, two tailed) between all values with asterisks and those for rats which did not receive any pretreatment and those for rats receiving MP- and Sap-preabsorbed immune vaginal fluid.

[b] Immune vaginal fluid was taken 7 days after a rechallenge from rats which cleared a primary *C. albicans* infection (7).

TABLE 3. Outcome of vaginal infection with *C. albicans* in rats treated with MAbs against MP and Sap

MAb[a]	Specificity	Mean no. of *C. albicans* vaginal CFU ± SE)[b] (no. of rats infected/total) on day:		
		0	1	10
None		>100 (5/5)	>100 (5/5)	30 ± 6 (4/5)
AF1	Antimannan	>100 (5/5)	37 ± 3* (5/5)	10 ± 2* (4/5)
4C8	Anti-MP (protein)	>100 (5/5)	>100 (5/5)	22 ± 2 (4/5)
8E11	Anti-MP (protein)	>100 (5/5)	>100 (5/5)	18 ± 10 (5/5)
GF1	Anti-Sap	>100 (5/5)	54 ± 8* (5/5)	2 ± 1* (4/5)

[a] Used in a single intravaginal administration 30 min after *C. albicans* challenge at a concentration of 100 μg of affinity-purified reagent per ml.

[b] Asterisks indicate a statistically significant difference ($P < 0.05$, two tailed) from vaginal CFU counts (expressed as 10^3 CFU/ml) of controls (rats given no MAb).

may have been induced by the primary *C. albicans* infection are unlikely to play a protective role.

Passive protection with anti-MP and anti-Sap MAbs. The data presented above prompted us to assess the effect of intravaginal administration of anti-MP and anti-Sap MAbs. For the former, three Abs were selected, two of which recognized distinct polypeptide epitopes (MAbs 4C8 and 8H11) and one of which recognized a mannan epitope (MAb AF1). Before use, the specificity of each MAb was rechecked by Western blotting. All MAbs were used at identical protein concentrations in a single intravaginal administration 30 min before challenge.

As shown in Table 3, both antimannan and anti-Sap MAbs, but not MAbs specific for polypeptide epitopes of MP, conferred early protection to rats (day 1) substantially comparable to that conferred by the passive administration of the immune vaginal fluid containing polyclonal anti-MP and anti-Sap Abs. Notably, the anti-Sap MAb, which is nonagglutinating and nonreactive with fungal cell surface, gave the same immediate protection (on day 1) as did the agglutinating antimannan MAb (>50% reduction of CFU counts; $P < 0.05$, two tailed). This protection was maintained at least until day 10, when only one rat of five given the anti-Sap MAb was still infected, with low *Candida* burden (Table 3).

Protection induced by vaccination with Sap2. Since, as shown above, both anti-Sap and antimannan Abs conferred protection against vaginal *Candida* challenge, and since it was previously shown that active immunization with a mannan extract conferred protection (7), we examined here whether protection could also be achieved by active immunization with the Sap antigen itself. The animals were therefore immunized by intravaginal administration of the enzyme in Freund's adjuvant; either unimmunized rats or rats given only Freund's adjuvant served as controls.

As shown in Fig. 1, rats administered the Sap preparation in adjuvant were indeed protected from vaginitis compared to rats given the adjuvant only or PBS. In Sap-immunized animals, specific anti-Sap Abs of IgG and IgA classes were elicited (data not shown).

Protective effects of pepstatin A. Since pepstatin is a well-known Sap inhibitor (39), we wondered whether treatment of *C. albicans*-infected rats with this substance would affect the rate of fungal clearance from the vagina. As shown in Table 4, treatment of infected rats with 100 μg of pepstatin induced about a >50% reduction of vaginal *Candida* burden during the experiment.

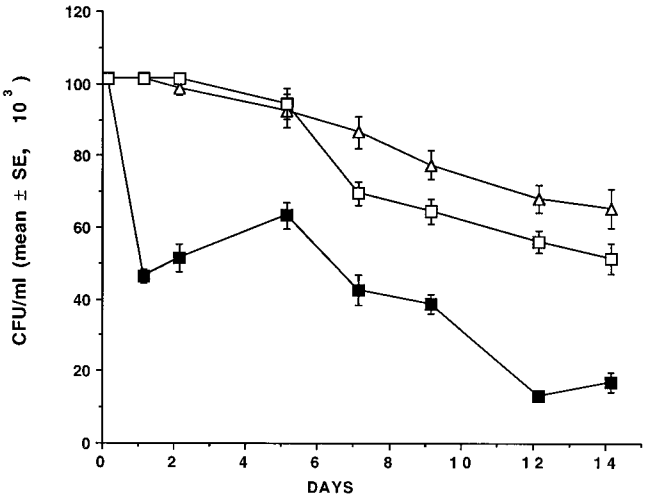

FIG. 1. Outcome of vaginal infection in oophorectomized, estradiol-treated rats immunized intravaginally with a Sap preparation in adjuvant (■) or given adjuvant alone (△) or PBS (□) before challenge with *C. albicans*. There were five rats per group. Starting day 1 postinfection, there was always a statistically significant difference ($P < 0.01$, two-tailed, Student's *t* test) between the numbers of vaginal CFU in the animals given Sap and those in rats given the adjuvant or PBS only (with the exception of CFU of Sap-immunized versus adjuvant- or PBS-treated rats on day 5, when the difference was significant at a *P* value of <0.05). The data are from one of two independent experiments which had similar results. SE, standard error.

DISCUSSION

Experimental models of vaginitis in rodents have recently been used to study the anti-*Candida* response and mechanisms of protection at the vaginal level (8, 21). Somewhat contrary to expectations based on clinical observations, these studies demonstrated that systemic CMI, although promptly elicited by vaginal *Candida* infection, has no effect on anticandidal protection at the vaginal level (17–20). As discussed by Fidel and Sobel (21) and by Cassone et al. (8), it seems more likely that a number of interplaying mechanisms, including local CMI and humoral immunity, may protect against vaginal infection by *Candida.*

Previous studies have shown that both adherence and Sap production are likely to exert a role in the mechanisms by which *C. albicans* infects and persists in the vagina (reviewed in reference 10). It has also been suggested that adherence and Sap may be interrelated in the expression of fungal pathogenicity (22). Another factor in the pathogenesis of vaginitis may

TABLE 4. Outcome of vaginal infection with *C. albicans* in rats treated with pepstatin

Treatment	Mean no. of *C. albicans* vaginal CFU ± SE on day[a]:			
	0	1	2	15
PBS	>100	>100	96 ± 4	58 ± 7
Pepstatin diluent[b]	>100	>100	>100	49 ± 3
Pepstatin[c]	>100	55 ± 1*	40 ± 6*	15 ± 2*

[a] Data are from one of two independent experiments (each with five rats per group) with similar results. Asterisks indicate a statistically significant difference ($P < 0.05$, two tailed) between vaginal CFU counts (data are expressed as 10^3 CFU/ml) of pepstatin-treated rats and those of controls (PBS or diluent treated).

[b] 1.9% (vol/vol) ethanol in water/0.1 ml (see below for the duration of treatment).

[c] Used at a concentration of 100 μg/ml in 1.9% ethanol in water solution and administered intravaginally 30 min after challenge, then daily for 7 days.

be the capacity of *C. albicans* to grow in a hyphal form, which seems to be associated with both the adherence and the expression of some members of the Sap family of isoenzymes (10, 25, 26). This form of growth may be important for pathogenicity because it may conceal immunodominant and protective *C. albicans* antigens, thus potentially conferring to the fungus the ability to escape from immune responses which are naturally elicited against the commensal yeast form (6, 10, 13, 21, 35, 44).

On the basis of these findings and the assumption that Abs could be among the humoral factors that play a role in anticandidal defense at the vaginal level, we found that passive transfer of vaginal fluids from *C. albicans*-immunized rats conferred protection to nonimmune rats and that this protection was associated with the Ig-containing fraction of the vaginal fluid (7). These results were strengthened by the independent discovery of candidacidal, protective, killer toxin-mimicking Abs naturally elicited in women with candidiasis or *C. albicans* carriage (37).

Although the immune vaginal fluid contained both anti-MP and anti-Sap Abs, and its protective ability was clearly reduced upon absorption on *C. albicans* yeast cells (7), our previous studies did not establish whether antimannan, anti-Sap, or other Abs with undefined specificity were responsible for the protection. Thus, we tried to establish more definitely the nature of the protective Abs by using selective adsorption and monoclonal reagents directed against the two putatively involved fungal antigens. We also examined whether the Ab response was directed against a T-dependent antigen.

The results obtained in the present investigation strongly suggest that both antimannan and anti-Sap Abs may play a role in the protection conferred by immune vaginal fluid. The Ab adsorption experiments demonstrated that the specificities of both Abs were indeed required for optimal protection, as neither Ab alone was sufficient to confer the same optimal level of protection given by the whole immune vaginal fluid. These experiments also showed that other undefined Abs are unlikely to participate in the protection, because no residual protection was observed in rats subjected to passive transfer of the vaginal fluid that had been preabsorbed with both MP and Sap.

The use of MAbs confirmed the above results. In fact, both antimannan and anti-Sap reagents conferred passive protection against the vaginal challenge. Two other MAbs directed against protein moieties of MP were ineffective, somewhat confirming that the protective anti-MP Ab needs to recognize a polysaccharide moiety. It would be of interest to establish the exact nature of this protective polysaccharide. Further studies with an expanded panel of MAbs with well-defined epitope specificities and a number of isotype-matched irrelevant MAbs as controls are required to clarify this issue.

Han and Cutler have recently reported the induction of Ab protective response by a mannan adhesin in a murine systemic *C. albicans* infection (24), in keeping with previous assumptions on the importance of Abs in controlling systemic candidiasis (33). An antimannan adhesin MAb (MAb B6.1) was also protective, and its protection was independent of agglutination (24). Whether MAb AF1 and MAb B6.1 recognize the same or related mannan epitopes has not been determined, although both epitopes are contained in chemically similar phosphomannoprotein complexes of *C. albicans* (6, 24, 27, 43, 44).

Another aspect of interest in this investigation is the capacity of a saccharide-free Sap preparation to confer active protection. The protection was coupled with specific Ab production and was presumably dependent on it, as inferred from the passive transfer of protection by anti-Sap Ab-containing vaginal fluids. The level of Sap-dependent protection was comparable to that previously obtained from active immunization with a mannan extract, overlapping the protection resulting from the clearing of the primary infection (7).

Also of interest are our findings that pepstatin A, a strong *C. albicans* Sap inhibitor (26, 39), reduced the level of vaginal infection by *C. albicans*. An anti-*Candida* effect of pepstatin has previously been reported by Ruchel et al. (39) in a model of murine-disseminated infections. Pepstatin may also affect adherence (1, 22), but this inhibitory activity is generally regarded as being mediated by the effect of pepstatin on Sap (26). More recently, Fallon and collaborators (16) have shown that pepstatin A reduces mortality by *C. albicans* in an intranasal inoculation model of murine-disseminated infection, somewhat confirming our previous assumptions about the role of Sap in mucosal rather than systemic infections (4, 11). All of these protective effects add to other indirect evidence favoring Sap's being a true virulence factor of *C. albicans* (4, 29, 38).

As for the different mannan molecules, it is difficult to define exactly the putative protective Sap antigen. Sap constitutes a family of at least eight homologous isoenzymes encoded by the *SAP1-SAP8* gene family (25, 26). We obtained some evidence that both *SAP1* and *SAP2* genes are expressed during experimental vaginal infection (14). The Sap preparation that we used for active immunization, detection of anti-Sap vaginal Abs, and MAb GF1 production is very likely to be the Sap2 isoenzyme, as it was obtained under induction and expression conditions typical for this isoenzyme (26, 38). It was purified as a single electrophoretic band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was shown to be carbohydrate free (38). Nonetheless, the homology between all isoenzymes of the family could imply that the protective epitopes might be common to the different Sap proteins, thus eliciting polyclonal Abs with a reactivity against most or all members of the Sap family. From this point of view, the identification of the epitope recognized by the anti-Sap MAb GF1 could be particularly important owing to the demonstrated protective ability of this MAb in vaginal infection.

The protective Abs proved to be directed against T-dependent antigens, as either no Ab response or an inconsistent Ab response against mannan or Sap was elicited by the primary *Candida* infection in nude rats and no protection was therefore conferred to these animals by the spontaneous clearing of the primary infection. While the T-cell dependence of the anti-Sap response is not surprising, that of the antimannan Ab response warrants some discussion. Mangeney et al. (31) demonstrated that in vitro antimannan Ab production is T-cell independent, type 2, meaning that T-cell-derived cytokines are needed for B-cell activation (42). Different from artificially prepared, deproteinated mannan extracts (5, 15, 31), our MP preparations contain covalently bound protein that makes a polysaccharide response most likely to be T dependent. One of these proteins (MP65) has recently been identified as a major target of the T-cell response in humans (23).

The T-cell dependence of the induction of anti-*Candida* Ab response and the consequent protection in rat vaginitis may provide a link between clinical observations which point to a role for CMI in the surveillance against mucosal and cutaneous forms of candidiasis (3, 21) and the results of our recent studies on the protective role of vaginal Abs (7, 8, 36, 37). We observed that athymic (nude) rats were unable to raise any consistent antimannan or anti-Sap Abs and were not protected against secondary infection, although they cleared the primary infection as efficiently as the euthymic animals. Our data suggest that an efficient CMI at the vaginal level may play a role in protection by providing the necessary help for the right Ab formation.

Previous clinical and immunological observations did not provide firm evidence for the presence of protective Abs in the vaginas of women after resolution of an infectious episode. In contrast, even elevated anti-*Candida* Ab titers were detected in some patients suffering from recurrent vaginitis attacks (21, 34). However, neither the specificity nor the isotype of these anti-*Candida* Abs (which did not confer protection and even enhanced infection) was determined in the above studies. It is not expected that every anti-*Candida* Ab is protective, especially in view of the *Candida* commensalism with humans and the expected plethora of irrelevant anti-*Candida* Abs (8). Despite this, at least one type of protective anti-*Candida* Ab has been recently found in the human vagina. This Ab mimicked in its function the yeast killer toxin and was probably directed against a transphyletic receptor (37). Recombinant anti-idiotypic Abs in the single chain format, mimicking the yeast killer toxin and binding to a *Candida* cell wall receptor, were also cytocidal and highly protective against rat candidal vaginitis (30). However, the protection reported in the present paper is not attributable to the anti-idiotypic Abs which were not found in the vaginal immune fluid used in this investigation. Moreover, the absorption experiments clearly demonstrated that the protection must be ascribed, at least predominantly, to anti-mannan and anti-Sap Abs.

Further studies need to address more specifically the nature of the protective epitopes as well as the mechanisms of anticandidal protection exerted by Abs at the vaginal level. It is important to establish whether these protective antigens and mechanisms are operating or being impeded in the human vagina. These studies may provide a valuable approach to the possible use of Abs (or their engineered derivatives) and *Candida* vaccines for a very widespread and sometimes difficult-to-treat human infection such as candidal vaginitis.

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REFERENCES

1. **Borg, M., and R. Rüchel.** 1988. Expression of extracellular acid proteinase by proteolytic *Candida* spp. during experimental infection of oral mucosa. Infect. Immun. **56:**626–631.
2. **Calderone, R. A., and P. C. Braun.** 1991. Adherence and receptor relationship of *Candida albicans*. Microbiol. Rev. **55:**1–20.
3. **Cantorna, M. T., and E. Balish.** 1990. Mucosal and systemic candidiasis in congenitally immunodeficient mice. Infect. Immun. **58:**1093–1100.
4. **Cassone, A., F. De Bernardis, F. Mondello, T. Ceddia, and L. Agatensi.** 1987. Evidence for a correlation between proteinase secretion and vulvovaginal candidosis. J. Infect. Dis. **156:**777–783.
5. **Cassone, A., A. Torosantucci, M. Boccanera, G. Pellegrini, C. Palma, and F. Malavasi.** 1988. Production and characterization of a monoclonal antibody to a cell surface, glucomannoprotein constituent of *Candida albicans* and other pathogenic *Candida* species. J. Med. Microbiol. **27:**233–238.
6. **Cassone, A.** 1989. Cell wall of *Candida albicans*: its functions and its impact on the host. Curr. Top. Med. Mycol. **3:**249–314.
7. **Cassone, A., M. Boccanera, D. Adriani, G. Santoni, and F. De Bernardis.** 1995. Rats clearing a vaginal infection by *Candida albicans* acquire specific, antibody-mediated resistance to vaginal reinfection. Infect. Immun. **63:**2619–2624.
8. **Cassone, A., S. Conti, F. De Bernardis, and L. Polonelli.** 1997. Antibodies, killer toxins and antifungal immunoprotection: a lesson from nature? Immunol. Today **18:**164–169.
9. **Chambers, W. H., N. L. Vujanovic, A. B. De Leo, M. W. Olskowy, R. B. Herbeman, and J. C. Hiserodt.** 1989. Monoclonal antibody to a triggering structure expressed on rat natural killer cells and adherent lymphokine activated killer cells. J. Exp. Med. **169:**1373–1389.
10. **Cutler, J. E.** 1991. Putative virulence factors of *Candida albicans*. Annu. Rev. Microbiol. **45:**187–218.
11. **De Bernardis, F., L. Agatensi, I. K. Ross, G. W. Emerson, R. Lorenzini, P. A. Sullivan, and A. Cassone.** 1990. Evidence for a role for secreted aspartate proteinase of *Candida albicans* in vulvovaginal candidiasis. J. Infect. Dis. **161:**1276–1283.
12. **De Bernardis, F., D. Adriani, R. Lorenzini, E. Pontieri, G. Carruba, and A. Cassone.** 1993. Filamentous growth and elevated vaginopathic potential of a nongerminative variant of *Candida albicans* expressing low virulence in systemic infection. Infect. Immun. **61:**1500–1508.
13. **De Bernardis, F., A. Molinari, M. Boccanera, A. R. Stringaro, R. Robert, J. M. Senet, G. Arancia, and A. Cassone.** 1994. Modulation of cell surface-associated mannoprotein antigen expression in experimental candidal vaginitis. Infect. Immun. **62:**509–519.
14. **De Bernardis, F., A. Cassone, J. Sturtevant, and R. Calderone.** 1995. Expression of *Candida albicans* SAP1 and SAP2 in experimental vaginitis. Infect. Immun. **63:**1887–1892.
15. **Domer, J. E.** 1989. *Candida* cell wall mannan: a polysaccharide with diverse immunologic properties. Crit. Rev. Immunol. **17:**33–51.
16. **Fallon, K., K. Bausch, J. Noonan, E. Huguenel, and P. Tamburini.** 1997. Role of aspartic proteases in disseminated *Candida albicans* infection in mice. Infect. Immun. **65:**551–556.
17. **Fidel, P. L., Jr., M. E. Lynch, and J. D. Sobel.** 1993. *Candida*-specific Th1-type responsiveness in mice with experimental vaginal candidiasis. Infect. Immun. **61:**4202–4207.
18. **Fidel, P. L., Jr., M. E. Lynch, and J. D. Sobel.** 1993. *Candida*-specific cell-mediated immunity is demonstrable in mice with experimental vaginal candidiasis. Infect. Immun. **61:**1990–1995.
19. **Fidel, P. L., Jr., M. E. Lynch, and J. D. Sobel.** 1994. Effects of preinduced *Candida*-specific systemic cell-mediated immunity on experimental vaginal candidiasis. Infect. Immun. **62:**1032–1038.
20. **Fidel, P. L., Jr., M. E. Lynch, D. H. Conaway, L. Tait, and J. D. Sobel.** 1995. Mice immunized by primary vaginal *Candida albicans* infection develop acquired vaginal mucosal immunity. Infect. Immun. **63:**547–553.
21. **Fidel, P. L., Jr., and J. D. Sobel.** 1996. Immunopathogenesis of recurrent vulvovaginal candidiasis. Clin. Microbiol. Rev. **9:**335–348.
22. **Ghannoum, M. A., and K. Abu Elteen.** 1986. Correlative relationship between proteinase production, adherence and pathogenicity of various strains of *Candida albicans*. J. Med. Vet. Mycol. **24:**407–413.
23. **Gomez, M. J., A. Torosantucci, S. Arancia, B. Maras, L. Parisi, and A. Cassone.** 1996. Purification and biochemical characterization of a 65-kilodalton mannoprotein (MP65), a main target of anti-*Candida* cell-mediated immune responses in humans. Infect. Immun. **64:**2577–2584.
24. **Han, Y., and J. E. Cutler.** 1995. Antibody response that protects against disseminated candidiasis. Infect. Immun. **63:**2714–2719.
25. **Hube, B., M. Monod, D. A. Schofield, A. J. P. Brown, and N. A. R. Gow.** 1994. Expression of seven members of the gene family encoding secretory aspartyl proteinases of *Candida albicans*. Mol. Microbiol. **14:**87–99.
26. **Hube, B.** 1996. *Candida albicans* secreted aspartyl proteinases. Curr. Top. Med. Mycol. **7:**55–69.
27. **Kanbe, T., and J. E. Cutler.** 1994. Evidence for adhesin activity in the acid-stable moiety of the phosphomannoprotein cell wall complex of *Candida albicans*. Infect. Immun. **62:**1662–1668.
28. **Klein, R. S., C. A. Harris, C. B. Small, B. Moll, M. Lesser, and G. H. Friedland.** 1984. Oral candidiasis in high-risk patients as the initial manifestation of the acquired immunodeficiency syndrome. N. Engl. J. Med. **311:**354–358.
29. **MacDonald, F., and F. C. Odds.** 1983. Virulence for mice of a proteinase secreting strain of *Candida albicans* and a proteinase-deficient mutant. J. Gen. Microbiol. **129:**431–438.
30. **Magliani, W., S. Conti, F. De Bernardis, M. Gerloni, D. Bertolotti, P. Mozzoni, A. Cassone, and L. Polonelli.** 1997. Therapeutic potential of antiidiotypic single chain antibodies with yeast killer toxin activity. Nat. Biotechnol. **15:**155–158.
31. **Mangeney, M., A. Fisher, F. le Deist, J. P. Latgé, and A. Durandy.** 1989. Direct activation of human B lymphocytes by *Candida albicans*-derived mannan antigen. Cell. Immunol. **122:**329–337.
32. **Mariani, M., M. Cianfriglia, and A. Cassone.** 1989. Is mouse IgM purification on protein A possible? Immunol. Today **10:**115–116.
33. **Mathews, R., and J. Burnie.** 1992. The role of hsp90 in fungal infection. Immunol. Today **13:**345–348.
34. **Mathur, S., G. Virella, J. Koistinen, E. O. Horger, A. Mahvi, and H. H. Fudenberg.** 1992. Humoral immunity in vaginal candidiasis. Infect. Immun. **15:**287–294.
35. **Mencacci, A., A. Torosantucci, R. Spaccapelo, L. Romani, F. Bistoni, and A. Cassone.** 1994. A mannoprotein constituent of *Candida albicans* that elicits different levels of delayed-type hypersensitivity, cytokine production, and anticandidal protection in mice. Infect. Immun. **62:**5353–5360.
36. **Polonelli, L., F. De Bernardis, S. Conti, M. Boccanera, M. Gerloni, G. Morace, W. Magliani, C. Chezzi, and A. Cassone.** 1994. Idiotypic intravaginal vaccination to protect against candidal vaginitis by secretory, yeast killer toxin-like anti-idiotypic antibodies. J. Immunol. **152:**3175–3182.
37. **Polonelli, L., F. de Bernardis, S. Conti, M. Boccanera, W. Magliani, M. Gerloni, C. Cantelli, and A. Cassone.** 1996. Human natural yeast killer toxin-like candidacidal antibodies. J. Immunol. **156:**1880–1885.

38. **Ross, I. K., F. de Bernardis, G. W. Emerson, A. Cassone, and P. Sullivan.** 1990. The secreted aspartate proteinase of *Candida albicans*: physiology of secretion and virulence of a proteinase-deficient mutant. J. Gen. Microbiol. **136:**687–694.
39. **Ruchel, R., B. Ritter, and M. Schaffrinski.** 1990. Modulation of experimental systemic murine candidosis by intravenous pepstatin. Zentralbl. Bakteriol. **273:**391–403.
40. **Sobel, J. D., G. Muller, and H. R. Buckley.** 1984. Critical role of germ tube formation in the pathogenesis of candidal vaginitis. Infect. Immun. **44:**576–580.
41. **Sobel, J. D.** 1985. Epidemiology and pathogenesis of recurrent vulvovaginal candidiasis. Am. J. Obstet. Gynecol. **152:**924–935.
42. **Stein, K. E.** 1992. Thymus-independent and thymus-dependent responses to polysaccharide antigens. J. Infect. Dis. **165**(Suppl. 1)**:**49–52.
43. **Torosantucci, A., C. Palma, M. Boccanera, C. M. Ausiello, G. C. Spagnoli, and A. Cassone.** 1990. Lymphoproliferative and cytotoxic responses of human peripheral blood mononuclear cells to mannoprotein constituents of *Candida albicans*. J. Gen. Microbiol. **136:**2155–2163.
44. **Torosantucci, A., C. Bromuro, M. J. Gomez, C. M. Ausiello, F. Urbani, and A. Cassone.** 1993. Identification of a 65-kDa mannoprotein as a main target of human cell-mediated immune response to *Candida albicans*. J. Infect. Dis. **168:**427–435.

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